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Iodometric Assay Method for Beta-Lactamase with Various Beta-Lactam Antibiotics as Substrates

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The rapid fixed-time assay for penicillinase was modified for measuring β-lactamase activity with twelve substrates, i.e., benzylpenicillin, ampicillin, cloxacillin, methicillin, carbenicillin, cefazolin, cephalothin, cephaloglycin, cephalexin, cephalosporin C, 7-aminocephalosporanic acid, and cefoxitin. The method depends upon the reduction of iodine by the hydrolyzed substrate. Determined experimentally, 1 mol of hydrolyzed penicillins consumed 3.4 to 4.0 mol of iodine (I_2). Iodine consumption of hydrolyzed cephalosporins varied widely from 1.7 for cephalothin to 3.7 for cefazolin. The method is useful for routine assay of β-lactamase activity with various substrates.

The rapid fixed-time assay for penicillinase devised by Sargent (2) is a simple and convenient method for routine use. The principle of the assay method is the same as that of Perret's iodometric method (1), depending upon the reduction of iodine by benzylpenicillin hydrolysis but measuring excess iodine colorimetrically instead of by titration with sodium thiosulfate. This method was originally used for the assay of penicillinase with benzylpenicillin as the only substrate.

Our studies on β-lactamases of gram-negative bacteria made it necessary to use various penicillins and cephalosporins as substrates. This report describes the modification of the rapid fixed-time assay method required for various β-lactam antibiotics.

MATERIALS AND METHODS

β-Lactamases. Four penicillinases and a cephalosporinase were used for enzymatic hydrolysis of β-lactam antibiotics. Three of the penicillinases used are mediated by R plasmids, i.e., RGN14, RGN823, and RGN238. These penicillinases were prepared from *Escherichia coli* strains harboring the respective R plasmids by methods described in previous papers (6, 8). The oxacillin-hydrolyzing penicillinase of *Aeromonas hydrophila* 67-P-24 was prepared by the procedure reported previously (4). Cephalosporinase was prepared from *Citrobacter freundii* GN346. Properties of the cephalosporinase were reported previously (3, 5).

Penicillins and cephalosporins. Penicillins and cephalosporins of established purities were kindly provided by the following pharmaceutical companies: benzylpenicillin, ampicillin, and cloxacillin, Meiji Seika Co., Tokyo, Japan; carbenicillin and cefazolin, Fujisawa Pharmaceutical Co., Osaka, Japan; cephalothin and cephaloglycin, Shionogi Pharmaceutical Co., Osaka, Japan; cephaloridine, cephalosporin C, and 7-aminocephalosporanic acid, Glaxo Laboratories Ltd., Greenford, Middlesex, England; methicillin, Beecham Research Laboratories, West Sussex, England; and cephalexin, Toyama Chemical Co., Tokyo, Japan; cefoxitin, Merck Sharp & Dohme Research Laboratories, Rahway, N.J.

Iodine reagent. Iodine reagent was prepared as described by Sargent for the original fixed-time assay method (2). A 5-ml portion of the iodine reagent contained 40 μmol of I_2 in acetate buffer (pH 4.0). The iodine reagent was prepared by adding 5 ml of stock iodine solution to 95 ml of the acetate buffer (80 g of anhydrous sodium acetate adjusted to pH 4.0 with acetic acid and brought to 2 liters with distilled water). The stock iodine solution contained 0.16 M iodine and 1.2 M potassium iodide (20.3 g of iodine and 100 g of potassium iodide dissolved in 500 ml of distilled water).

Spectrophotometric assay of β-lactamase activity. The standard procedure for assay of hydrolyzed β-lactam antibiotic is about the same as that described by Sargent (2). β-Lactamase in 2.5 ml of 0.1 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.0) was preincubated at 30°C for 5 min in an assay tube to which 0.5 ml of the substrate solution was added with rapid mixing. After incubation at 30°C for an appropriate period, the enzyme reaction was stopped by adding 5 ml of the iodine reagent with rapid mixing. After standing at room temperature for 10 min (30 min for cefazolin), the absorbance of the mixture at 540 nm (green filter) was measured with a Klett-Summerson colorimeter.

Two blank tests were carried out. Blank A contained 3 ml of the phosphate buffer and 5 ml of the iodine reagent and gave a reading of 420 Klett units at 540 nm. Blank B contained the phosphate buffer and the substrate solution, but no enzyme, and was incubated under conditions identical to those of the assay tube, except that the enzyme was added after the iodine reagent.

The amount of substrate hydrolyzed in the assay tube was calculated from the following formula:

$$\text{Hydrolyzed substrate (micromoles)} = \frac{\Delta Ku}{\text{Ku-blank A}} \times \frac{40}{F} \quad (1)$$

The activity of the enzyme sample tested was calculated from the following formula:

$$\beta\text{-Lactamase activity (units per milliliter)} = \frac{\Delta Ku}{\text{Ku-blank A}} \times \frac{40}{F} \times \frac{1}{T} \times \frac{1}{V} \quad (2)$$

In these equations, ΔKu = (Ku-blank B) minus (Ku-sample). Ku-blank A, Ku-blank B, and Ku-sample, absorbance is expressed as Klett units; F, consumed moles of iodine (I_2) per mole of the hydrolyzed substrate; T, time (minutes) for enzyme reaction; V, volume (milliliters) of enzyme solution added to the assay tube. One unit of enzyme activity was defined as that quantity which hydrolyzes 1 μmol of substrate per min under the conditions used.

Hydrolysis of penicillins and cephalosporins. The β-lactam ring of β-lactam antibiotics was quantitatively hydrolyzed by enzymatic and alkaline hydrolysis. Penicillinase from *E. coli* RGN14 was used for hydrolysis of benzylpenicillin and ampicillin. Penicillinase from *E. coli* RGN823 was used for hydrolysis of carbenicillin. Cloxacillin and methicillin were hydrolyzed by the oxacillin-hydrolyzing penicillinase prepared from *E. coli* RGN238 or *A. hydrophila* 67-P-24. Cephalosporinase from *C. freundii* GN 346 was used for hydrolysis of cephaloridine, cephalothin, cephalexin, cefazolin, and cephalosporin C. The β-lactam ring of the above antibiotics was also cleaved by alkaline hydrolysis.

7-Aminocephalosporanic acid, cephaloglycin, and cefoxitin are resistant to hydrolysis by many β-lactamases. Accordingly, the standard cleavage products of these cephalosporins were prepared by alkaline hydrolysis.

Enzymatic hydrolysis. A 5-ml portion of 0.1 M phosphate buffer (pH 7.0) containing 100 μmol of β-lactam antibiotic and an adequate amount of the enzyme was mixed and incubated at 30°C. A portion (300 μl) of the reaction mixture was removed at different intervals, and the reaction was stopped by addition of phosphate buffer and 5 ml of the iodine reagent. The final volume of the mixed solution was 8 ml. After the solution stood for 10 min at room temperature, its optical density was measured at 540 nm. Hydrolysis of the substrate led to a decrease in absorbance. After the substrate was completely hydrolyzed, the absorbance became constant (Fig. 1). The solution of hydrolyzed substrate was chilled in an ice bath, and used immediately.

To confirm the complete hydrolysis of the antibiotic, the remaining antibacterial activity was determined by the cup-diffusion test with *Bacillus subtilis* ATCC 6633 as the indicator organism (7). No antibacterial activity could be detected, indicating that less than 1% of the initial concentration of antibiotic remained. Before bioassay, β-lactamase was inactivated at 74°C for 15 min in the case of cephalosporinase and 30 min in the case of penicillinase. No appreciable hydrolysis of the antibiotics occurred during this heat treatment.

Alkaline hydrolysis. A 5-ml portion of a solution containing 100 μmol of β-lactam antibiotic in 0.2 N sodium hydroxide was incubated at 30°C. A portion (300 μl) of the solution was removed at varying intervals and placed in an ice-bath neutralized with 300 μl of 0.2 N hydrochloric acid after which 2.4 ml of phosphate buffer was added. Iodine consumption of the neutralized solution was assayed, and hydrolysis of the antibiotic was confirmed by bioassay.

RESULTS

Kinetics of the iodometric assay. Benzylpenicillin and cephalothin were hydrolyzed by varying concentrations of β-lactamase or bacterial cells possessing the enzymes. The change in absorbance was linear and correlated with concentrations of the enzyme samples until at least 80% of the iodine added was consumed by the hydrolyzed substrates. With different reaction times and a constant amount of enzyme, the change in absorbance was also linear and, for the first 60 min of incubation, correlated with the length of the reaction period.

Determination of F values for various penicillins and cephalosporins. F, defined as the number of iodine (I_2) molecules consumed by the hydrolyzed substrate under appropriate conditions, was determined experimentally for each β-lactam antibiotic. The standard hydrolyzed substrates were prepared by the procedures described above. The kinetics of the hy-

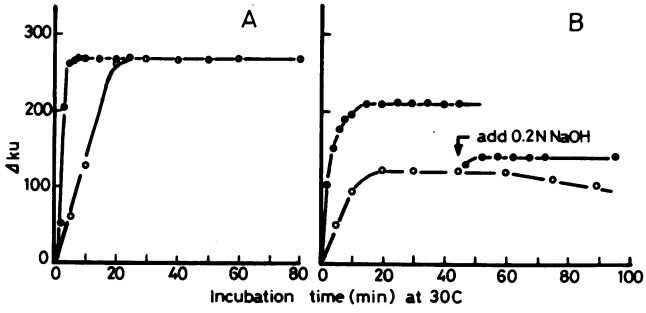

FIG. 1. *Hydrolysis of benzylpenicillin and cephalothin by β-lactamases or alkali. (A) Benzylpenicillin was hydrolyzed by the penicillinase of E. coli RGN14* (○) *or in 0.2 N sodium hydroxide* (●). *(B) Cephalothin was hydrolyzed by the cephalosporinase of C. freundii* GN346 (○) *or in 0.2 N sodium hydroxide* (●).

drolysis of benzylpenicillin and cephalothin, as examples, are shown in Fig. 1. β-Lactam antibiotics, listed in Table 1, except cephaloglycin, 7-aminocephalosporanic acid, and cefoxitin, were subjected to both enzymatic and alkaline hydrolysis. The same *F* values were obtained with the two methods, except in the case of cephalothin. A representative result is shown in Fig. 1A. In the case of cephalothin, the product of alkaline hydrolysis gave a different *F* value, about 1.8 times that obtained by enzymatic hydrolysis. After cephalothin was completely hydrolyzed by the enzyme, addition of alkali caused a slight increase in *F* value (Fig. 1B). The β-lactam rings of cephaloglycin, 7-aminocephalosporanic acid, and cefoxitin, which are resistant to hydrolysis by many β-lactamases, were hydrolyzed by alkali.

Artificial reaction mixtures containing exactly defined amounts of hydrolyzed substrate were prepared and reacted with the iodine reagent, and decolorization followed as a function of time. The results obtained with benzylpenicillin and cephalothin, as examples, are shown in Fig. 2. Absorbance is expressed as the percentage of that found 10 min after addition of the iodine reagent. In the case of penicillins, decolorization was almost complete 5 min after the addition of the iodine reagent (Fig. 2A). This suggested that the reaction between penicilloic acid and iodine is complete within 10 min. However, in the case of cephalosporins, decolorization increased with time (Fig. 2B). This was most marked in the case of cefazolin (Fig. 3). *F* values for five penicillins and seven cephalosporins, calculated from data obtained from experiments similar to those shown in Fig. 2 and 3, are listed in Table 1.

Hydrolyzed cefazolin consumed much more iodine than other cephalosporins, yielding an *F* value of 3.7 10 min after addition of the iodine reagent, 4.5 at 30 min, and steadily larger up to 60 min. The most linear correlation between the amount of the hydrolyzed substrate and the enzyme concentration was observed when the iodine consumption was measured 30 min after addition of the reagent.

On the basis of *F* values, the amount of each substrate in an assay tube, equivalent to about 70 μmol of iodine (I_2), was determined to be as follows: benzylpenicillin, 7 mg; ampicillin, 7 mg;

TABLE 1. *Iodine consumption of the hydrolyzed β-lactam antibiotics*

Agent	Range of substrate concn[a] (μmol)	Mean of *F* values[b] (range)
Benzylpenicillin	1.0–8.0	4.0 (3.7–4.2)
Ampicillin	1.0–8.0	3.7 (3.3–3.8)
Carbenicillin	2.0–6.0	3.4 (3.3–3.6)
Cloxacillin	1.0–8.0	3.7 (3.6–3.8)
Methicillin	1.0–8.0	3.9
Cephalosporin C	2.0–14.0	1.8 (1.7–2.0)
Cephalothin	2.0–14.0	1.7 (1.5–1.9)
Cefazolin	1.8–9.1	3.7 (3.3–3.9)
Cefazolin[c]	1.8–9.1	4.5 (3.9–4.9)
Cephalexin	1.9–9.4	3.0 (2.9–3.0)
7-Aminocephalosporanic acid	2.0–14.0	2.5 (2.3–2.8)
Cephaloglycin	2.0–12.0	2.7 (2.7–2.8)
Cefoxitin	2.0–14.0	1.9 (1.7–2.1)

[a] The amount of the hydrolyzed substrate in the assay tube. All the hydrolyzed substrates were prepared by enzymatic hydrolysis except those for 7-aminocephalosporanic acid, cephaloglycin, and cefoxitin, which were obtained by alkaline hydrolysis.

[b] *F* values represent moles of iodine (I_2) consumed per mole of the hydrolyzed substrate, as measured 10 min after mixing with the iodine reagent.

[c] *F* values measured 30 min after mixing with the iodine reagent are shown together with 10-min values for cefazolin.

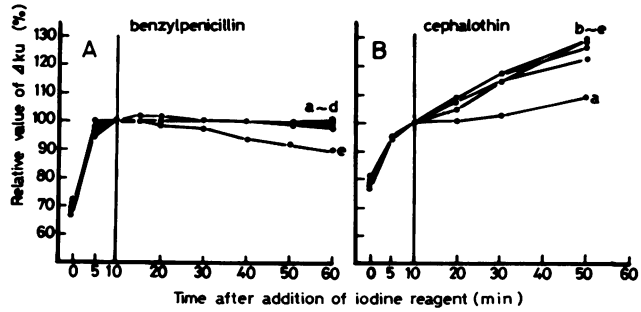

FIG. 2. *Kinetics of iodine consumption by hydrolyzed benzylpenicillin and cephalothin. At zero time, 3 ml of phosphate buffer containing a defined amount of the enzymatic hydrolysate of benzylpenicillin or cephalothin was mixed with 5 ml of the iodine reagent. Absorbance at 540 nm was followed at room temperature, and the value of ΔKu was calculated. Blank B contained the same number of micromoles of intact substrate. Iodine consumption was expressed as the percentage of the ΔKu value 10 min after addition of the iodine reagent. (A) The amounts of hydrolyzed benzylpenicillin in the reaction mixture were 1, 2, 4, 6, and 8 μmol (see a to e, respectively). (B) The amounts of the hydrolyzed cephalothin were 4, 6, 8, 10, and 14 μmol (see a to e, respectively).*

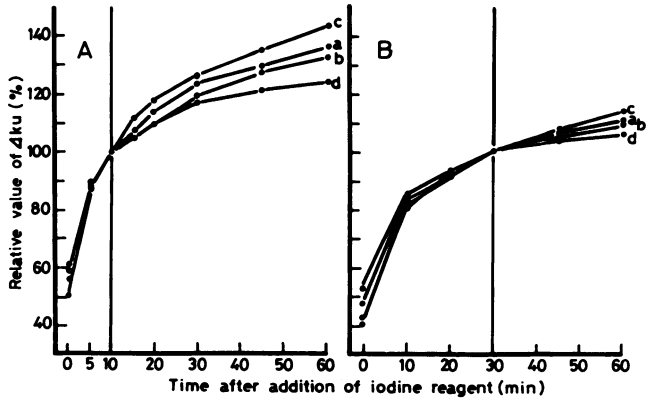

FIG. 3. *Kinetics of iodine consumption by the hydrolyzed cefazolin. Experimental procedures were the same as in Fig. 2. The amounts of the hydrolyzed cefazolin were 1.8, 3.6, 5.4, and 9.1 μmol (see a to d, respectively). Iodine consumption was expressed as the percentage of the ΔKu value 10 min (A) or 30 min (B) after addition of the iodine reagent.*

carbenicillin, 9 mg; cloxacillin, 9 mg; methicillin, 8 mg; cephalosporin C, 18 mg; cephalothin, 17 mg; cefazolin, 7 mg; cephalexin, 8 mg; 7-aminocephalosporanic acid, 8 mg; cephaloglycin, 11 mg; and cefoxitin, 16 mg.

Cephaloridine is the most common substrate for assay of cephalosporinase activity. However, hydrolyzed cephaloridine produces a muddy material with iodine, making colorimetric assay impossible.

DISCUSSION

Experiments described in this paper suggest that the iodometric method is applicable to various β-lactam antibiotics if the *F* value for each antibiotic and suitable assay conditions are employed. *F* values of penicillins varied little, from 3.4 to 4.0. *F* values of cephalosporins varied more widely, from 1.7 to 3.7. Moreover, the iodine consumption of cephalothin and cefazolin increased with the length of the reaction period. Such a phenomenon was common to other cephalosporins (data not shown). Therefore, a definite time for reaction with iodine, as well as the appropriate *F* value for each substrate, is necessary for the iodometric assay method. We suggest that 10 min is adequate for the reaction time with iodine, except for cefazolin for which a reaction time of 30 min may be suitable.

As shown in Fig. 1B, the *F* value for hydrolyzed cephalothin depended upon the hydrolysis procedure. This fact suggests that alkaline and enzymatic hydrolysis of some β-lactam antibiotics led to different products. We measured the *F* values of 7-aminocephalosporanic acid, cephaloglycin, and cefoxitin by alkaline hydrolysis because of their resistance to β-lactamases. The possibility cannot be neglected that these *F* values may not be identical with those obtained with enzymatic cleavage.

The iodometric assay method presented in this paper has the following disadvantages: extremely crude enzyme samples give a turbidity greater than 450 Klett units (blank B), and the assay method cannot be applied to cephaloridine. Measurements in such cases can be made with the iodometric method of Perret (1). The values for β-lactamase activity obtained with both the present method and that of Perret were compared. When benzylpenicillin, ampicillin, carbenicillin, cloxacillin, cephalothin, cephalexin, and cefazolin were used as substrates, the value obtained with the two methods agreed within 15%. In our experience, the number of enzyme samples that could be handled by the Perret method within a definite time period was less than one-fifth that accommodated by the colorimetric method.

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